Characterisation of normal peripheral blood cells in cycle identified by monoclonal antibody Ki-67

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Abstract

Aims: **To investigate the numbers, morphology, and lineage assignment of Ki-67 positive cells in peripheral blood from normal subjects.**

Methods: **Single and double immunoenzymatic staining procedures, immunoperoxidase, and immunoalkaline phosphatase were used with Ki-67, a monoclonal antibody that recognises a nuclear antigen present in proliferating cells, with markers expressed in B and T lymphocytes and monocytes.**

Results: **In the five healthy donors 2·1% (range 1·6–3·7%) cells of the blood mononuclear fraction and 2·7% (range 2·3–3·9%) lymphocytes were Ki-67 positive. Of these, 88% (range 85–90%) were small cells and 12% (range 10–15%) were medium sized. Forty one per cent of the Ki-67 positive cells were CD3 positive by double immunoenzymatic staining and corresponded to T lymphocytes, and 11·4% were mature B cells expressing κ or λ light chains. Monocytes detected by the anti-lysozyme antibody were consistently Ki-67 negative. Half of the Ki-67 positive lymphocytes could not be accounted for by B or T cells with the markers used. Most Ki-67 positive cells were of small size; the B lymphocytes in cycle showed abundant cytoplasm and features suggestive of lymphoplasmacytic differentiation.**

Conclusions: **The methodology described is useful for the simultaneous detection of nuclear and cytoplasmic antigens. The demonstration that a proportion of normal blood lymphocytes are in cell cycle raises the issue of whether immunophenotypic analysis of Ki-67 positive cells in haemopoietic malignancies with peripheral blood disease should be carried out to define more precisely the proportion of normal and neoplastic cells in cycle.**

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The proportion of cells in cycle in normal and pathological tissues used to be estimated by the number of mitotic figures seen on histological examination. New techniques have permitted more precise characterisation of the dynamics of this process. Methods widely used include ^{3}H thymidine uptake into the cellular DNA, measured by autoradiography or scintillation counting,[1] assessment of the DNA content with propidium iodide or acridine orange by cytofluorometry,[2 3] and the reactivity with autoantibodies from patients with systemic lupus erythematosus or leukaemia which detect nuclear antigens expressed in proliferating cells.[4 5] Monoclonal antibodies against the transferring receptor (OKT9), 5-bromo-2' deoxyuridine, and the proliferating cell nuclear antigen (PCNA), have also been used.[6–8] Among the antibodies that detect cycling cells, Ki-67 seems to be the most widely used. Ki-67 is a mouse antibody raised against the human L428 Hodgkin's disease cell line that recognises a nuclear antigen expressed in proliferating cells but not in resting cells.[9] This antigen is expressed throughout the G1, S, G2 and M phases of the cell cycle but is absent in G0 phase.[10] Because there is little information on the reactivity with Ki-67 in non-neoplastic conditions, we investigated the numbers, morphology, and lineage assignment of Ki-67 positive cells in peripheral blood from normal subjects using single and double immunoenzymatic staining procedures.

Methods

Peripheral blood, taken into heparin, was obtained from five healthy adults, aged 24–45 years. Cytospin slides were prepared from mononuclear cells separated with Ficoll-Hypaque on a Shandon cytocentrifuge, air dried overnight, wrapped in aluminium foil and stored at −20°C until immunostaining.

Ki-67 was used to detect proliferating cells and UCHT1 (CD3) to detect T lymphocytes. Rabbit polyclonal antibodies to human light chains (R anti-κ and R anti-λ) and to human lysozyme (R anti-Lys) were used to identify mature B cells and myelomonocytic cells, respectively. Second and third layer reagents comprised: peroxidase-conjugated goat anti-mouse immunoglobulins (G anti-M); monoclonal mouse-peroxidase-antiperoxidase (PAP) complexes; alkaline phosphatase-conjugated swine anti-rabbit immunoglobulins (S anti-R); rabbit anti-mouse immunoglobulins (R anti-M); and monoclonal mouse alkaline phosphatase-anti-alkaline phosphatase (APAAP) complexes. Normal mouse and rabbit sera were used as controls, replacing the relevant monoclonal and polyclonal antibodies. All the reagents were purchased from Dakopatts, Buckinghamshire, England, except UCHT1 which was a gift from Professor PCL Beverley.

SINGLE IMMUNCYTOCHEMICAL STAINING

Immediately before immunostaining the cytospins were warmed at room temperature,

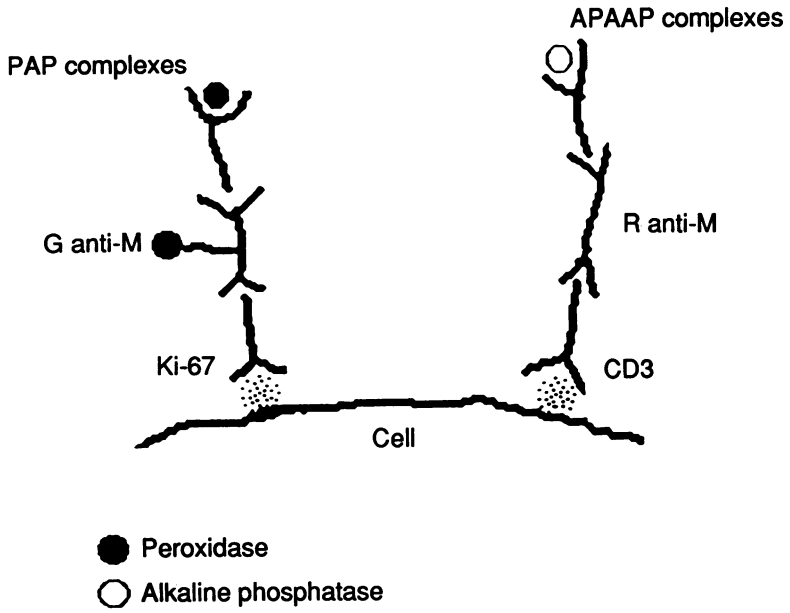

Figure 1 Diagram of double immunoenzymatic staining with monoclonal antibodies Ki-67 and anti-CD3.
(G anti-M: peroxidase-conjugated goat anti-mouse immunoglobulins; PAP: monoclonal mouse peroxidase-anti-peroxidase complexes; R anti-M: rabbit anti-mouse immunoglobulins; APAAP: monoclonal mouse alkaline phosphatase-anti-alkaline phosphatase complexes.)

unwrapped, and fixed for 10 minutes in acetone. Immunocytochemical staining with Ki-67 was performed using a modified indirect immunoperoxidase sandwich technique.[11] Briefly, cytospins were incubated for 30 minutes with Ki-67 diluted 1 in 25 in phosphate-buffered saline (PBS), followed by a peroxidase-conjugated G anti-M (diluted 1 in 20 in PBS with 10% normal human AB serum) for 30 minutes. After a wash in PBS, PAP complexes (diluted 1 in 70 in PBS) were added to increase the staining intensity. The peroxidase reaction was then carried out by incubating for 10 minutes in a developing medium containing PBS, diaminobenzidine tetrahydrochloride 0·6 mg/ml (Sigma), and 0·01% of hydrogen peroxide. Cells were counterstained with haematoxylin S 'Gurr' (Merck) for one minute. All the incubations were performed at room temperature. Normal mouse serum in place of the first layer was used as a negative control.

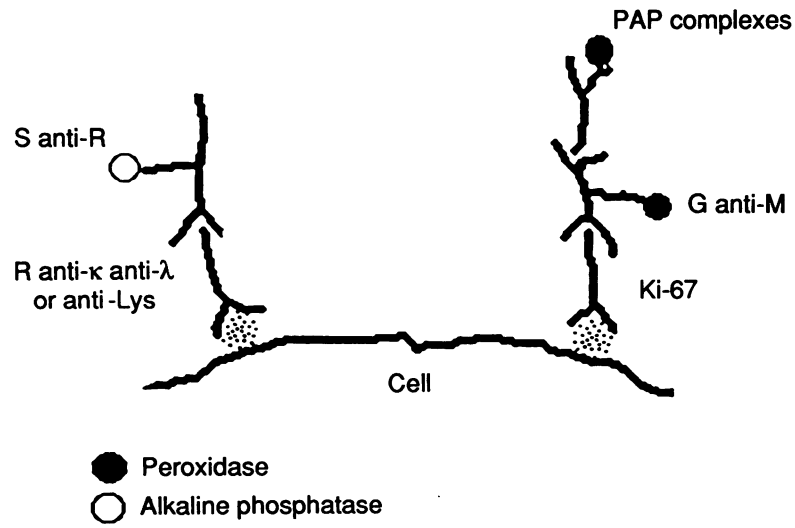

Figure 2 Diagram of double immunoenzymatic staining with polyclonal and monoclonal antibodies.
(R anti-κ, anti-λ or anti-Lys: rabbit polyclonal antibodies to human κ or λ light chains and to human lysozyme; S anti- R: alkaline phosphatase-conjugated swine anti-rabbit immunoglobulins.)

DOUBLE IMMUNOCYTOCHEMICAL STAINING

Ki-67 and anti-CD3

For the simultaneous detection of Ki-67 and CD3, sequential immunoenzymatic staining was applied that combined the immunoperoxidase sandwich with the immunoalkaline phosphatase technique[12 13] (fig 1). After staining with Ki-67 the cytospins were washed for five minutes in TRIS-buffered saline (TBS) and subsequently incubated with the UCHT1 (diluted 1 in 10 in TBS) for 30 minutes at room temperature. This was followed by a 30 minute incubation with R anti-M (diluted 1 in 20 in TBS with 5% normal human AB serum) and after a further wash with the APAAP complexes (diluted 1 in 60 in TBS) for 45 minutes. The developing medium for the alkaline phosphatase containing Fast Red salt as substrate.[13] Slides were counterstained with haematoxylin for one minute and mounted with Glycergel (Dakopatts). Normal mouse serum replaced UCHT1 in the negative control.

Ki-67 and anti-light chains Ig

Polyclonal antibodies to human light chains R anti-κ and R anti-λ were used in an indirect immunoenzymatic sandwich before or simultaneously with the immunoperoxidase staining for Ki-67 (fig 2). In the sequential procedure cytospins were incubated with the primary polyclonal R anti-κ (diluted 1 in 1300 in TBS) or R anti-λ (diluted 1 in 1500 in TBS) for 30 minutes at room temperature, followed by an alkaline phosphatase-conjugated S anti-R (diluted 1 in 20 in TBS with 5% normal human AB serum) for 30 minutes. The alkaline phosphatase reaction was performed using the same substrate referred to above and, after a five minute wash with PBS, the Ki-67 immunoperoxidase staining was carried out. Slides were counterstained with haematoxylin for one minute and mounted with Glycergel. In the simultaneous procedure the reagents for the two sequences were mixed at each incubation step and all the incubations were carried out for 30 minutes. Normal mouse serum replaced Ki-67 in the negative control.

Ki-67 and anti-lysozyme

To combine Ki-67 with the antibody R anti-Lys sequential or simultaneous double immunoenzymatic staining was performed (fig 2). In the sequential procedure the immunoperoxidase staining with Ki-67 was carried out first, and after a five minute wash with TBS cytospins were incubated with R anti-Lys (diluted 1 in 250 in TBS). The subsequent steps were carried out as described for the anti-light chain antibodies. In the simultaneous procedure the reagents for the two sequences were mixed at each incubation step and all the incubations were carried out for 30 minutes. Normal rabbit serum was used instead of R anti-Lys as a negative control.

Table 1 Incidence, absolute number, and cell size of Ki-67 positive cells in normal peripheral blood

Sample	Age/sex	Ki-67 positive cells in: Mononuclear fraction (%)	Ki-67 positive cells in: Lymphocyte population (%)	Absolute number of Ki-67 positive cells $\times 10^9/l$	Size: Small (%)	Size: Medium (%)
1	41/F	2·8	2·9	0·079	88*	12
2	29/F	2·1	2·4	0·052	85	15
3	24/F	3·7	3·9	0·098	86	14
4	45/M	2·0	2·3	0·043	89	11
5	41/M	1·6	2·7	0·113	90	10

*The size was calculated within the Ki-67 positive population.

EVALUATION OF THE PERCENTAGE AND MORPHOLOGY OF PROLIFERATING CELLS

The proportion of Ki-67 positive cells was evaluated by light microscopy with an oil-immersion objective (magnification × 1000) examining an average of 2000 mononuclear cells per sample. The proportion of double stained cells was evaluated at × 400 magnification by counting 200 Ki-67 positive cells. The mean (SEM) was calculated on a minimum of three different counts on the same or different slides. The median and range were used to report the results of the five normal samples. Ki-67 positive cells were considered to be small when they were comparable with the size of small lymphocytes (10–12 μm), of medium size when they were twice the size of a small lymphocyte, and large when they were three times the size of a small lymphocyte.

Results

PROLIFERATING CELLS IN NORMAL BLOOD

The proportion, absolute number, and size of Ki-67 positive cells in the mononuclear fraction and within the lymphoid population are shown in table 1. In the five samples 2·1% (range 1·6–3·7%) Ki-67 positive cells were found in the mononuclear fraction and 2·7% (range 2·3–3·9%) among the lymphocyte population. The absolute number of the Ki-67 positive cells was 0·079 × 10^9/l (range 0·043–0·113%).

According the size, 88% (range 85–90%) of Ki-67 positive cells were small, with a round or slightly indented nucleus and scanty cytoplasm. The remaining 12% (range 10–15%) Ki-67 positive cells were of medium size, with a round or "kidney" shaped eccentric nucleus and abundant cytoplasm.

Intense brown nuclear staining and good preservation of morphological details were obtained. The reaction was always confined to the nucleus and no diffuse cytoplasmic staining was observed. The few nucleolated cells present showed a stronger stain in the nucleolus, particularly at its periphery.

LINEAGE ASSIGNMENT OF KI-67 POSITIVE CELLS

The percentages of Ki-67 positive cells co-expressing the T, B, and myelomonocytic markers are shown in table 2. The Ki-67 positive cells that showed strong cytoplasmic reactivity with CD3 comprised 41·0% (range 37·9–49·5%). Of these Ki-67/CD3 positive cells, 96% (range 93–92%) were small, with a round or indented nucleus and scanty cytoplasm and 4% (range 3–7%) were of medium size, with more abundant cytoplasm. The staining with CD3 was often localised, close to the nuclear indentation (fig 3).

Of the Ki-67 positive cells, 6·7% (range 3·9–16·5%) showed strong cytoplasmic reactivity with anti-κ and 4·7% (range 2·7–13·4%) with anti-λ. Of the Ki-67/κ positive cells, 58% (range 40–62%) were small and 42% (range 38–60%) medium sized. Of the Ki-67/λ positive cells, 46% (range 25–73%) were small and 54% (range 27–75%) medium sized. Both small and medium sized Ki-67 positive cells showed morphological features of lymphoplasmacytic cells, with a round slightly ecentric nucleus, abundant cytoplasm, and well developed Golgi zone that was more evident in the larger size cells (fig 4).

All the cells showing cytoplasmic reactivity with anti-lysozyme (monocytes) were Ki-67 negative (fig 5). Half the Ki-67 positive cells could not be accounted for by the B and T cell markers used.

The cell morphology was always well preserved and showed good contrast between the nuclear brown reaction of Ki-67 and the cytoplasmic red staining of anti-CD3, anti-κ, anti-λ and anti-Lys. When the two primary antibodies used in the double staining were raised in different species, no cross-reactivity or differences of staining intensity were found when the reagents of each sandwich were applied simultaneously.

PERCENTAGE OF MATURE T AND B CELLS POSITIVE FOR KI-67

The percentages of T and B lymphocytes

Table 2 Mean (SEM) percentage of double stained cells within Ki-67 positive population

Sample	Ki-67/CD3	Ki-67/κ	Ki-67/λ	Ki-67/Lys
1	41·0 (2·2)	10·3 (1·6)	6·7 (0·7)	0
2	41·8 (1·0)	3·9 (0·1)	4·7 (1·0)	0
3	37·9 (3·2)	16·5 (2·2)	13·4 (2·4)	0
4	49·5 (0·9)	4·6 (0·4)	2·7 (0·1)	0
5	38·9 (0·7)	6·7 (0·3)	3·8 (0·4)	0

Table 3 Percentage of mature T and B cells positive for Ki-67

Sample	T cells	B cells*
1	1·5	2·2
2	1·3	0·9
3	1·9	5·3
4	1·5	0·6
5	1·2	2·0

*κ positive + λ positive.

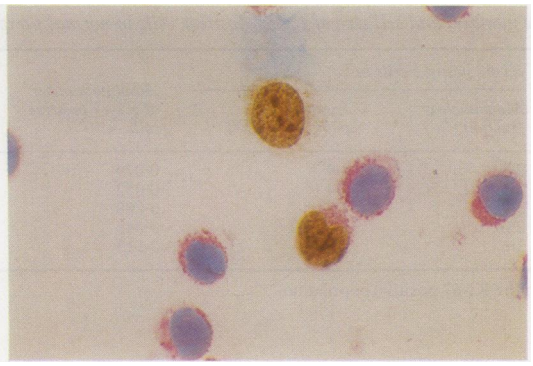

Figure 3 Normal blood lymphocytes positive with CD3 (red cytoplasmic stain with APAAP). Note that two lymphocytes are Ki-67 positive (brown nuclear deposit) and that one of them also expresses CD3.

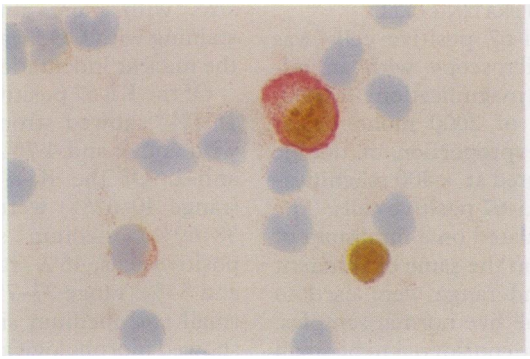

Figure 4 A Ki-67 positive medium size lymphocyte (brown nuclear deposit) with eccentric nucleus and abundant cytoplasm containing κ light chain (red deposit) with features suggestive of lymphoplasmacytic differentiation. Note a Ki-67 positive/κ negative small cell and a Ki-67 negative/κ positive lymphocyte.

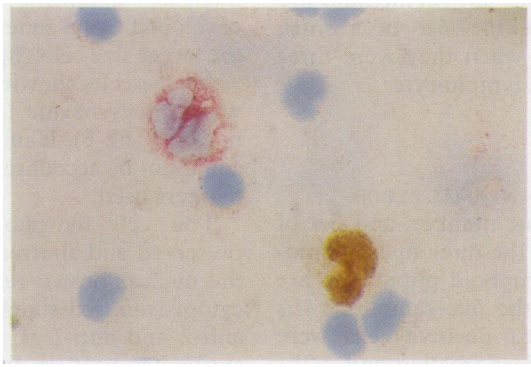

Figure 5 A "monocytoid" looking cell positive with Ki-67 (brown nuclear deposit) but anti-Lys negative. Note that there is a monocyte strongly anti-Lys positive (red cytoplasm) but negative with Ki-67.

which were positive for Ki-67 are shown in table 3. Among CD3 positive lymphocytes, 1·5% (range 1·2–1·9%) were Ki-67 positive and among mature B cells (κ+ and λ+), 2·0% (range 0·6–5·3%) were Ki-67 positive.

Discussion

Ki-67 recognises a nuclear antigen present in proliferating cells but is absent in resting cells.[9 10] Since the first report in 1983, this monoclonal antibody has been widely used to assess the proliferation rate in a variety of malignant tumours including haemopoietic malignancies. The percentage of cells in cycle seems to have prognostic value and is relevant to the choice of effective treatments used.[14–19] Few studies have analysed the expression of Ki-67 in non-neoplastic conditions, particularly in normal blood mononuclear cells.

An early report by Gerdes *et al* suggested that normal blood lymphocytes and monocytes are Ki-67 negative.[9] In contrast, Ki-67 positive lymphoid cells have been shown in tonsils, normal infant and adult bone marrow, and thymus.[20–22] In addition, Ki-67 is always expressed in normal blood lymphocytes after 72 hours of culture with the mitogen phytohaemagglutinin.[21]

We estimated the percentage, morphology, and lineage assignment of Ki-67 positive cells in peripheral blood from normal subjects by single and double immunoenzymatic staining, using markers for circulating B and T lymphocytes and monocytes. In contrast to published data[9] we have shown the presence of a small population of proliferating cells in the blood from normal subjects, ranging from 1·6% to 3·7% of the mononuclear fraction. We have also shown that a significant proportion of the Ki-67 positive cells correspond to T and B lymphocytes with monocytes being consistently Ki-67 negative. Within T and B lymphoctes the proportion of Ki-67 positive cells represents only a minority—1·5% of CD3 positive lymphocytes and 2·0% of mature B cells.

Most Ki-67 positive lymphocytes were small in size, with a round or slightly indented nucleus and scanty cytoplasm; a few were medium sized with an eccentric nucleus and abundant cytoplasm. Some of the medium sized cells had a "monocytoid" appearance. The monocytic nature of these cells, however, was excluded by double immunoenzymatic staining as they were always anti-lysozyme negative. Thus while most Ki-67/CD3 positive cells were small, more than 50% of the Ki-67 positive cells expressing immunoglobulin light chains were medium sized. These small and medium sized Ki-67 positive B lymphocytes showed a round eccentric nucleus and abundant cytoplasm, a morphological appearance suggestive of late B cells with plasmacytic differentiation.

This double immunoenzymatic staining procedure seems to be useful in identifying simultaneously nuclear and cytoplasmic antigens. The morphology was well preserved and the labelling strong and permanent, permitting easy detection of Ki-67 positive cells. The potential problem of cross-reactivity was solved by using antibodies raised in different species—rabbit for the detection of B cells and monocytes and mouse for Ki-67. It was also possible to mix the reagents of the two reactions at each incubation and the only extra time required to complete the procedure was that needed for the development of the second enzyme label. Furthermore, the cytoplasmic staining with the rabbit polyclonal antibodies was strong and easily visualised compared with the weak staining of mouse antibodies. During double immunoenzymatic labelling the peroxidase staining is always developed before the alkaline phosphatase because of the tendency of the peroxidase reaction to mask antigenic sites on the first monoclonal antibody.[13] According to this procedure, good results were obtained with the combination of Ki-67/CD3 and Ki-67/Lys. When the Ki-67 reaction was developed before the anti-κ and anti-λ staining, cross-reactivity was always observed between the two reactions (data not shown). This was probably due to the reaction of the anti-light chain antibodies with the light

chains of the second and third layers of the immunoperoxidase sandwich. This problem was solved by labelling the cells with the anti-light chain antibodies first or at the same time as the Ki-67 staining. We did not observe cross-reactivity when combining the two mouse monoclonal antibodies Ki-67 and UCHT1, as previously described.[13]

Several authors have reported that the percentage of Ki-67 positive cells is an indicator of the biological behaviour of a tumour and therefore has important prognostic and therapeutic implications.[23 24] The demonstration that a proportion of normal blood cells are in cycle raises the issue of whether immunophenotypic analysis of the Ki-67 positive cells in haemopoetic malignancies with peripheral blood disease should always be carried out to define more precisely the proportion of normal and neoplastic cells in cycle.

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